Detection and characterization of cytoplasmic hepatitis B virus reverse transcriptase

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It was recently found that the Duck hepatitis B virus (DHBV) reverse transcriptase is primarily a non-encapsidated cytoplasmic molecule that is rapidly translated and has a very short half-life. Here, a non-encapsidated reverse transcriptase from the human Hepatitis B virus (HBV) was characterized. HBV polymerase accumulated in the cytoplasm in a manner similar to non-encapsidated DHBV polymerase. However, the HBV polymerase accumulated at an apparently lower concentration and had a longer half-life than the DHBV enzyme, and it displayed no evidence of the post-translational modifications observed for DHBV. Unlike the DHBV polymerase, immunofluorescence detection of the HBV polymerase in cells was suppressed by the core protein, and this suppression occurred independently of encapsidation. This implies an interaction between the polymerase and core in addition to encapsidation, but the polymerase and core did not co-immunoprecipitate, so the interaction might not be direct. These data indicate that production of cytoplasmic, non-encapsidated polymerase is conserved among the hepadnaviral genera. Furthermore, conservation of the cytoplasmic form of the polymerase suggests that it might have function(s) in virus replication or pathology beyond copying the viral genome.

INTRODUCTION

Hepadnaviruses are small enveloped DNA viruses that infect birds, rodents and primates. Human Hepatitis B virus (HBV) chronically infects over 250 million people worldwide and is a major cause of hepatitis, cirrhosis and hepatocellular carcinoma (Hollinger & Liang, 2001). Although hepadnaviruses are DNA viruses, they replicate by reverse transcription of an RNA copy of the genome [the pregenomic RNA (pgRNA)] within cytoplasmic particles composed of the viral core protein (C). Reverse transcription is catalysed by a virally encoded reverse transcriptase (P) (Ganem & Schneider, 2001; Summers & Mason, 1982).

Encapsidation is the process in which the pgRNA and P are incorporated specifically into nascent viral core particles. In this ordered process, P binds to an RNA stem–loop (ε) at the 5′ end of the pgRNA (Bartenschlager et al., 1990; Hirsch et al., 1990; Junker-Niepmann et al., 1990) and then capsid form, presumably through polymerization of C protein dimers around the P:pgRNA complex. The HBV capsid contains 120 dimers of C in an icosahedral arrangement (Bottcher et al., 1997; Crowther et al., 1994), this ‘core particle’ serves to protect the genome from hazards such as nucleases and to provide an optimal environment for reverse transcription.

The pgRNA is not only an intermediate in DNA replication, but it is also a bicistronic mRNA that encodes C and P. Although the P open reading frame (ORF) is downstream of the C ORF on the pgRNA, it is translated by de novo initiation from its own AUG codon, rather than by frameshifting such as is employed by retroviruses (Chang et al., 1989; Fouillot & Rossignol, 1996; Hwang & Su, 1999; Lin & Lo, 1992; Ou et al., 1990; Schlicht et al., 1989). The unusual bicistronic structure of the pgRNA and difficulties in detecting P by enzymic or physical methods led to the impression that P was inefficiently translated and rapidly encapsidated. This in turn led to the belief that P did not accumulate to detectable levels outside core particles.

However, we found recently that the Duck hepatitis B virus (DHBV) P is translated rapidly, is encapsidated inefficiently, accumulates to easily detectable levels in the cytoplasm and is degraded rapidly (Yao et al., 2000, 2003; Yao & Tavis, 2003). Therefore, DHBV P is primarily a short-lived cytoplasmic protein. Coupled with the extremely limited genetic capacity of the hepadnaviruses, this observation led us to speculate that P may have additional roles in virus replication or pathology beyond replication of the viral genome, such as regulating cellular or viral processes (Yao et al., 2000).

Although DHBV and HBV are both hepadnaviruses with highly similar genetic organizations and replication cycles, the existence of HBV P as a short-lived cytoplasmic protein
cannot be assumed because DHBV is an *Avihepadnavirus* while HBV is an *Orthohepadnavirus*, and hence there are significant differences between the viruses. DHBV produces two fewer proteins than does HBV (DHBV lacks the middle surface antigen and the X protein), there is very little primary sequence identity between the two viruses and DHBV is non-pathogenic in its natural host whereas HBV is a major human pathogen. Therefore, we investigated whether HBV produces a cytoplasmic, non-encapsidated form of its reverse transcriptase similar to that of DHBV, and if so, how its biochemical characteristics compared with non-encapsidated DHBV P.

**METHODS**

**Viruses and plasmids.** pCMV-HPol contains the HBV strain adw2 P gene, with mutations that ablate expression of the large and small surface proteins (Bruss & Gamem, 1991) and X protein (C2804T), cloned downstream of the human cytomegalovirus (CMV) promoter in pCDNA3.1-Zeo+ (Invitrogen). pCMV-HPol expresses P with the proline in position 2 altered to alanine resulting from optimizing the P Kozak sequence. pCMV-HPol− is a negative control in which P expression was ablated by inserting stop codons after the first and second ATGs. pHBV1.5-LE− (HBV1.5) is a greater-than-genome-length expression vector for the HBV genome carrying the mutations that ablate the large and small surface glycoproteins. pCMV-HBV-LE− (CMV-HBV) and its derivatives are greater-than-genome-length expression vectors containing 1-2 copies of the HBV (adw2) genome carrying the surface antigen mutations downstream of the CMV promoter cloned into pBS− (Promega). pCMV-HBV-LEC− is pCMV-HBV-LE− with a stop codon in the C ORF after nt 98. pCMV-HBV-LEe− contains mutations that ablate the bulge of the 5′ e and block encapsidation (Pollack & Gamem, 1993). pCMV-HBV-LECe− contains both the C and e mutations.

**Cell culture, transfection and isolation of intracellular HBV cores.** Huh7 (human hepatoma) and HepG2 (human hepatoblastoma) cells were maintained in Dulbecco’s modified Eagle’s medium/F12 with 10% fetal bovine serum. Transfections employed FuGENE (Roche Diagnostics) according to the manufacturer’s instructions. HBV cores were isolated from Huh7 or HepG2 cells transfected with pCMV-HBV-LE− by lysis in core particle preparation lysis buffer [CPLB; 10 mM Tris (pH 7.5), 1 mM EDTA, 0.25% NP40, 50 mM NaCl, 8% sucrose] followed by sedimentation through a 30% sucrose cushion as described (Tavis et al., 1998).

**Immunoprecipitation.** Transfected Huh7 cells were lysed in 0.75× radioimmunoprecipitation assay buffer [RIPA; 1× RIPA is 20 mM Tris (pH 7.2), 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 2-7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4] plus 0-5% NP40 and 10% glycerol for 10 min on ice. Anti-HBV P antibodies [rabbit polyclonal antibodies against aa 1–198 or mouse monoclonal antibodies (zu Putlitz et al., 1999) or anti-HBV C antibodies (Austral Biologicals)] were bound to protein A/G beads (Calbiochem), and the antibody-bead complexes were incubated with cell lysates overnight. The immunocomplexes were washed four times with 1 ml of the appropriate buffer, and P was released by boiling in Laemmli buffer. Following SDS-PAGE, radioactive P was detected and quantified by phosphorimager analysis.

**Metabolic labelling and pulse–chase determination of half-life of P.** Transfected Huh7 cells were washed twice with Dulbecco’s modified Eagle’s medium lacking methionine and cystine (labeling medium) and pulsed with labeling medium supplemented with 120 μCi (4-44 MBq) [35S]methionine/cysteine ml−1 (EasyTag Express; PerkinElmer Life Sciences) and 1% fetal bovine serum. For pulse–chase experiments, cells were labelled for 1 h, rapidly washed twice with Dulbecco’s modified Eagle’s medium/F12, and then fed with Dulbecco’s modified Eagle’s medium/F12 containing 10% fetal bovine serum. The transition from the labelling to chase periods was performed as rapidly as possible (typically under 2 min), and all media were equilibrated to 37°C and 5% CO2 before use.

**Southern and Western Blots.** Southern blotting was performed as described (Staprans et al., 1991) with internally 32P-labelled monomeric HBV DNA as a probe and detected by phosphorimager analysis. Western blots were performed as described (Yao et al., 2000) employing commercial anti-HBV C antibodies (Austral Biologicals).

**Immunofluorescence.** Huh7 cells were grown on glass coverslips and transfected with pCMV-HPol, pCMV-HPol−, pCMV-HBV-LE−, pCMV-HBV-LEC−, pCMV-HBV-Lee−, or pBS−. Two days post-transfection, cells were fixed with 3-7% formalin in PBS for 10 min at room temperature followed by ice-cold methanol for 7 min and were blocked by incubation with PBS containing 5% goat serum at 37°C for 50 min. Primary and secondary antibodies were diluted in PBS/5% goat serum, and incubated for 2 h or 50 min, respectively. Coverslips were washed three times in PBS. Standard immunofluorescence images were captured digitally at 600× with a SPOT camera attached to an Olympus fluorescence microscope. Confocal microscopy was performed at a magnification of 600× on a Bio-Rad MRC 1024 confocal system attached to a Nikon Optiphot microscope.

**Digital images.** Digital images were processed with Adobe Photoshop and assembled in Adobe Illustrator.

**RESULTS**

**Detection of non-encapsidated HBV P**

Huh7 and HepG2 cell lines produce infectious HBV upon transfection with the viral genome (Sells et al., 1987; Yaginuma et al., 1987), and are the standard models for HBV replication in culture. We transfected Huh7 cells with expression constructs for HBV P or the HBV pgRNA, metabolically labelled the cells with [35S]methionine/cysteine, lysed the cells in 0.75× RIPA and immunoprecipitated P using a rabbit polyclonal antibody that reacts with the HBV P terminal protein domain (aa 1–198). HBV P was detectable when expressed directly from the CMV promoter (Fig. 1, lane 2) and when the entire HBV pgRNA was expressed from the CMV promoter (Fig. 1, lane 3). The latter construct produces authentic pgRNA that directs production of reverse transcription-competent core particles (data not shown). However, we were only intermittently able to detect P when the pgRNA was expressed from the native HBV promoters in a greater-than-genome-length construct (Fig. 1, lane 4). The lower level of P, in this case, was consistent with the much lower production of core particles from this construct (data not shown). HBV P also could be directly immunoprecipitated from HepG2 cells transfected with plasmids expressing P or the pgRNA from the CMV promoter (data not shown).

To determine whether immunoprecipitated P is associated
with C or with core particles, Huh7 cells were transfected with the CMV-driven pgRNA construct (pCMV-HBV) or a negative control pgRNA construct (pCMV-HBV-LEC−), in which C expression was ablated. The cells were metabolically labelled with [35S]methionine/cysteine, lysed and immunoprecipitated with anti-HBV P antibodies. As a positive control, [35S]-labelled P was translated in vitro, diluted into a mock-transfected lysate and immunoprecipitated (lane 5).

To exclude the possibility that the P we detected by direct immunoprecipitation came from core particles that were disrupted by the lysis buffers, we performed two experiments. First, we isolated cytoplasmic HBV core particles from transfected Huh7 cells employing the mild lysis buffer CPLB. The core particles were incubated in either CPLB, 0–75 × RIPA or PEB at 4°C overnight to mimic the immunoprecipitation conditions and were sedimented through a 20% sucrose gradient. The core particles in the pellet were then detected by anti-C Western blot. Fig. 3(a) reveals that the large majority of the HBV C in the core particle preparation could be resedimented following incubation in the lysis buffers, indicating that it remained particulate under these conditions. Second, to exclude the possibility that the presence of C in the resedimentation pellets was due to formation of aggregates of disrupted core particles or disrupted core particles that remained large enough to sediment by themselves, we suspended isolated core particles in 1× DNase I buffer (Promega), 0–75 × RIPA or PEB, and incubated them with DNase I at 37°C for 2 h. If the core particles were disrupted enough by the
buffers to release P (~90 kDa) or to permit entry of the IgGs used for immunoprecipitation (~150 kDa), they would permit entry of DNase I (31 kDa), and hence the viral DNA would become sensitive to DNase I digestion. Southern blot analysis of DNAs purified from core particles treated in this manner revealed that the viral DNA remained resistant to DNase digestion (Fig. 3b), although naked plasmid DNA incubated under identical conditions was efficiently digested (Fig. 3c).

Together, these data indicate that (i) HBV P can be immunoprecipitated directly from cellular lysates, (ii) immunoprecipitable P is not stably associated with C and that our lysis conditions did not disrupt core particles enough to release P or permit entry of the immunoprecipitating antibodies. Therefore, the HBV P that can be immunoprecipitated directly is not encapsidated, and thus is similar to the non-encapsidated DHBV P we characterized previously (Yao et al., 2000, 2003; Yao & Tavis, 2003). However, detecting non-encapsidated HBV P is more difficult than is detecting DHBV P. Although some of this difficulty may be due to the sensitivity of our anti-HBV P antibodies, this is unlikely to be the only explanation because similar levels of HBV P were detected when anti-HBV P monoclonal antibodies produced by the Wands and Lanford labs (zu Putlitz et al., 1999) were employed (data not shown). Therefore, we feel that HBV P probably accumulates to lower levels in cultured cells than does DHBV P.

**Half-life of non-encapsidated HBV P**

The half-life of P was determined by transfecting Huh7 cells with expression vectors for P and performing pulse-chase experiments 3 days after transfection. Transfected cells were metabolically labelled with [35S]methionine/cysteine for 1 h, washed with non-radioactive medium, supplied with non-radioactive medium and incubated for various times. At each time point, cells were lysed, P was immunoprecipitated and radioactivity in P was quantified by phosphorimage analysis. The half-life of HBV P expressed from pCMV-HPol was 87 +/− 8 min (Fig. 4), and the half-life was unchanged when P was expressed from the pgRNA in the presence or absence of C (i.e. from pCMV-HBV-LE− or pCMV-LEC−; data not shown). Therefore,
non-encapsidated HBV P is considerably more stable than the DHBV P, whose half-life varies from 15–25 min depending on the time post-transfection (Yao & Tavis, 2003).

Intracellular localization of HBV P

The intracellular distribution of HBV P was assessed by immunofluorescence. Expressing P from the CMV promoter in transfected Huh7 cells revealed extensive granular staining throughout the cytoplasm (Fig. 5, panel c). P was not detected when transfected cells were stained with an irrelevant antibody (panel b) or when non-transfected cells were stained (panel a). Huh7 cells transfected with HBV P were stained simultaneously with anti-P antibodies and markers for the endoplasmic reticulum, mitochondria, Golgi apparatus or proteasomes, and the cells were analysed by dual-channel confocal immunofluorescence microscopy. P, ERP72 (endoplasmic reticulum-resident chaperone), cytochrome C (mitochondria), GP73 (Golgi marker; Kladney et al., 2000) and the 20S proteasomal subunit x5 (Affinity Bioreagents) were all distributed in the cytoplasm (nucleus and cytoplasm for the x5 subunit), but the merge of the datasets revealed little co-localization between P and the organelle markers, except possibly some co-localization with the endoplasmic reticulum (data not shown). This is very similar to our observations with DHBV P (Yao et al., 2000).

Expressing the pgRNA from the CMV promoter (pCMV-HBV) and staining cells simultaneously for C and P yielded many cells with a very strong signal for C but only a few cells that were weakly positive for P (Fig. 6a, panel ‘C, P, ε’). However, when we ablated core protein expression from the pgRNA vector, anti-P staining became much stronger (panel ‘P, ε’). In contrast to the large difference in the ability to detect P from the pgRNA in the presence or absence of C by immunofluorescence, metabolic labelling and immunoprecipitation revealed that very similar amounts of P were made in the two cultures (Fig. 6b, lanes 2 and 3). Similar results were obtained with monoclonal antibody 2C8 that recognizes the amino-terminal domain of HBV P (zu Putlitz et al., 1999). Therefore, co-expression of HBV P and C from the pgRNA greatly reduced the ability to detect P in cells by immunofluorescence with antibodies directed against the terminal protein domain of P. Importantly, encapsidation did not mask detection of P by immunofluorescence because when encapsidation was blocked by ablating ε on the pgRNA, P was still difficult to detect by immunofluorescence (Fig. 6a, compare panels ‘C, P, ε’ and ‘C, P’) although equivalent amounts of P were present (Fig. 6b, lanes 2 and 4). Therefore, expression of HBV P and C in the same cell greatly reduces the ability to detect P by immunofluorescence through a mechanism that is independent of encapsidation. This implies an interaction between the two proteins, but the interaction may not be direct because we have been unable to co-immunoprecipitate C with P (Fig. 2).

Unlike HBV, the intracellular distribution and detection sensitivity of DHBV P is unaffected by the presence of C. However, DHBV expression is analysed in LMH chicken hepatoma cells (Condreay et al., 1990). To exclude the possibility that the effect of HBV C on the ability to detect P was due to differences in cell types rather than differences between the viruses, we transfected wild-type and C-DHBV pgRNA expression vectors into Huh7 cells and performed dual-channel immunofluorescence for DHBV P and C. As in LMH cells, DHBV P could be detected by immunofluorescence with equal efficiency in the presence or absence

![Fig. 4. Pulse–chase determination of the half-life of non-encapsidated P. Huh7 cells were transfected with pCMV-HPol or pCMV-HPol− as a negative control, briefly metabolically labelled with [35S]methionine/cysteine, incubated in non-radioactive medium for the indicated times, lysed and P was immunoprecipitated. P was detected and quantified by phosphorimager analysis.](http://vir.sgmjournals.org)

![Fig. 5. HBV P is a cytoplasmic protein. Huh7 cells were transfected with pCMV-HPol and P was detected by immunofluorescence with polyclonal rabbit anti-HBV P antibodies. (a) Non-transfected cells stained with anti-HBV P. (b) Cells transfected with pCMV-HPol and stained with anti-DHBV P antibodies as a negative control. (c) Cells transfected with pCMV-HPol and stained with anti-HBV P antibodies. Panels (a) and (b) are standard immunofluorescence images and (c) is a confocal image.](http://vir.sgmjournals.org)
of C in Huh7 cells (data not shown). Therefore, the masking effect of HBV C on P is due to viral differences, not cell-specific factors.

**DISCUSSION**

Immunoprecipitation of proteins from Huh7 and HepG2 cells transfected with HBV pgRNA expression constructs revealed the existence of cytoplasmic P in which the amino terminus is accessible to antibodies. We conclude that the fraction of P we detected by direct immunoprecipitation is not encapsidated because none of the lysis buffers we employed (CPLB, PEB or 0.75 × RIPA) disrupted the core particles (Fig. 3), there is no DNA attached to directly precipitated P (data not shown) and we have been unable to co-immunoprecipitate C with anti-P antibodies (Fig. 2). This is very similar to our observation with non-encapsidated DHBV P (Yao et al., 2000, 2003; Yao & Tavis, 2003). Therefore, like DHBV P, HBV P appears to be produced in excess over the amount needed for production of viral capsids, and hence these data argue against the translation rate of P being rate limiting for encapsidation, as has been proposed (Bartenschlager & Schaller, 1992). These results also imply that cytoplasmic P is probably the main source of antigen that stimulates the early, vigorous immune responses to P, which can be as rapid and as strong as the responses to C, a major viral structural protein (Feitelson et al., 1988; Kann et al., 1993; Rehermann et al., 1995; Weimer et al., 1990).

The biochemical characteristics of non-encapsidated P from DHBV and HBV are similar but not identical (Table 1). Similarities include a granular distribution in the cytoplasm without obvious co-localization with the endoplasmic reticulum or Golgi apparatus. Differences include an apparently lower accumulation level and a longer half-life for HBV P. Furthermore, non-encapsidated HBV P migrates at its predicted mass, without the slower mobility isoforms found for DHBV P on denaturing SDV-PAGE (Yao et al., 2000, 2003). The slower mobility isoforms presumably result from post-translational modification, and hence we have no evidence for extensive post-translational modification of HBV P. An additional difference is that co-expression of HBV C with P in Huh7 cells greatly reduces the sensitivity of detection of P by immunofluorescence, despite equivalent expression of P as determined by immunoprecipitation (Fig. 6). In contrast, no effect of DHBV C on P can be detected beyond encapsidation itself.

The immunofluorescence experiment in Fig. 6 reveals that HBV C somehow masks the epitopes on P to which our

![Fig. 6. C masks P independently of encapsidation. Huh7 cells were transfected with pBS- (vector), pCMV-HBV-LE- (C, P, ε), pCMV-HBV-LEC- (P, ε), pCMV-HBV-LEe- (C, P) or with pCMV-HBV-LECe- (P). (a) Cells were fixed, stained simultaneously with anti-C and anti-P antibodies and C and P were detected by dual-channel immunofluorescence. (b) Cells from duplicate plates were metabolically labelled with [35S]methionine/cysteine, lysed and P was immunoprecipitated.

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<th>Characteristic</th>
<th>DHBV</th>
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<td>Amount</td>
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antibodies bind, but the masking does not involve encapsidation because masking is apparent even when encapsidation is blocked by ablation of e. We have been unable to co-immunoprecipitate C with anti-P antibodies (Fig. 2), so we have no evidence for a direct interaction of P and C in this effect. However, we cannot exclude the possibility of a direct interaction between P and C because co-immunoprecipitation is a very stringent assay, and many biologically relevant interactions are not stable enough to survive co-immunoprecipitation.

Our data indicate that non-encapsidated P accumulates in the cytoplasm, but do not preclude possible interactions between P and the exterior of capsids. However, if P is associated with the exterior of capsids, our data indicate that the binding is too weak to survive immunoprecipitation (Fig. 2). Lott et al., (2000) have reported extensive interactions between HBV P and C when the proteins are expressed in insect cells from baculovirus vectors, or in Huh7 cells when the proteins are expressed from vaccinia virus vectors. We have not detected these complexes, even when using identical lysis conditions. However, our experiments employed P and C expressed from the pgRNA and used anti-HBV P or C antibodies instead of epitope-tagged P and anti-FLAG antibodies, and so the differing results could be attributable to the alternate techniques employed.

HBV P has been previously detected in the nucleus of diseased livers by immunofluorescence (McGarvey et al., 1996). We were unable to detect unambiguously HBV P by immunofluorescence in 26 paraffin-embedded HBV-diseased livers by immunofluorescence (McGarvey et al., 1990). Other HBV genes are known to have dual roles: the C ORF encodes the C protein, which provides human liver tissues and Nandini Sen for assistance in generating anti-HBV polymerase antibodies. This work was supported by NIH grants CA91327 and AI38447.

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